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Characterization of components of photosynthetic machinery during oxidative stress-induced programmed cell death in Pea (*Pisum sativum)*

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By

Mansi Bhatt

(Reg. No. UIAR/10811)

Supervisor

Dr. Budhi Sagar Tiwari

Professor

Department of Biotechnology & Bioengineering

Institute of Advanced Research, Gandhinagar, Gujarat-India

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Ph.D. Thesis Synopsis

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Name of Student	Mansi Bhatt
Ph.D. Registration No.	UIAR/10811
Supervisor and Co supervisor	Dr. Budhi Sagar Tiwari, Professor, Department of Biotechnology & Bioengineering Institute of Advanced Research, Gandhinagar, Gujarat-India.

TABLE OF CONTENT

Sr. No.	Content	Page No.
1.	Abstract	1
2.	Introduction	2
3.	Objectives	4
4.	Material and Methods	4
5.	Results	8
6.	Discussion	11
7.	Conclusions and Summary	13
8.	References	15
9.	Paper Published/communicated	19
10.	Conferences/Symposia attended	20

1. Abstract

A cellular characteristic of redox processes is the generation of reactive oxygen species (ROS). Since ROS contribute to post-translational changes and destroy nucleic acids, they are generally detrimental to the cell. Antioxidant enzymes and non-enzymes are often used by cells to detoxify the harmful effects of ROS. However, when the amount of ROS surpasses the limit of antioxidant detoxification capacity, the target undergoes either necrotic or programmed cell death. It has been demonstrated that when the cellular ROS pool is moderate, cells choose to die by programmed cell death (PCD). The plant PCD process has not been thoroughly studied, whereas the animal PCD pathway has been well-depicted. The presence of chloroplasts, which are known to produce singlet oxygen, adds complexity and intrigue to the study of plant PCD. We have demonstrated the role of chloroplasts in regulating abiotic stress-induced PCD in our earlier correspondence. The function of cytochrome f (cyt f) in the singlet oxygen-enriched cellular environment and its interacting partners downstream in the PCD execution event have been investigated in the current work. Through the production of cyt f, which ultimately interacts with the components of the proteasomal pathway and is followed by certain ATG proteins, we have demonstrated in our study that enrichment of singlet oxygen leads challenged plants to undertake the PCD process.

2.Introduction

Reactive species of oxygen (ROS) are continuously produced by metabolic processes in plant cellular compartments that harbours redox reactions (Tripathy & Oelmüller, 2012). The cellular ROS pool's amplitude and quantum increase when a plant experiences stress (Schieber & Chandel, 2014). Antioxidant enzymes and non-enzyme substances that are naturally present in cells mitigate the harmful effects of ROS under normal and regulated development settings (Juan et al., 2021). However, the production of these reactive compounds increases to a point where it exceeds the antioxidant machinery of the cell when exposed to disturbed environmental conditions. The fate of challenged cells varies according on the amount of cellular ROS that is increased. Necrotic cell death occurs when cellular ROS levels are high, whereas moderate increases cause cell death, although they usually follow a controlled pathway (Ambastha et al., 2020; Rodríguez-Serrano et al., 2012; Sachdev et al., 2021). Cellular ROS, on the other hand, participate in signaling processes that promote plant growth and development at a low level (Ali et al., 2023).

Among the several ROS produced in plant cells, chloroplasts frequently produce singlet oxygen ($^1O_2$) through a type II photosensitization reaction that occurs when an electron is transferred from an excited chlorophyll molecule to the closest accessible oxygen molecule (Dmitrieva et al., 2020). Protoporphyrin IX, Mg-protoporphyrin IX, and protochlorophyllide (Pchlide) are membrane-bound excited chlorophyll biosynthesis intermediates that undergo photodynamic reactions to produce $^1O_2$ in addition to photosensitization reactions of excited chlorophyll molecules. As these intermediates are connected to the plastid membrane, upon illumination, these molecules become excited but cannot transfer the absorbed energy to the corresponding acceptor and return to their ground state. During the transition back to the ground state, the energy released interacts with molecular oxygen, producing $^1O_2$ through a type II photosensitization process (Bassi & Dall'Osto, 2021; Dmitrieva et al., 2020). Once $^1O_2$ is produced within a cell, it impacts cellular functions since the cell lacks the enzymatic ability to counteract the toxic effects of $^1O_2$ (Tripathy & Oelmüller, 2012). Nevertheless, certain

chloroplast-localized antioxidant compounds such as tocopherol and carotenoids can effectively quench singlet oxygen, thus partially mitigating the cellular damage caused by $^1O_2$.

The regulatory function of reactive oxygen species (ROS) in the regulation of programmed cell death (PCD) has been clearly demonstrated in both plants and animals (Ye et al., 2021). However, the role of chloroplasts in enhancing the ROS levels within the cell and its impact on PCD remains less clearly defined in plants. In our prior study, we demonstrated that an active chloroplast is necessary for the occurrence of salinity-induced PCD in green tissues (Ambastha et al., 2017). Furthermore, we established the involvement of chloroplast-generated singlet oxygen ($^1O_2$) in executing the PCD process during oxidative stress in Arabidopsis (Ambastha et al., 2020). To gain insights into the mechanistic aspects of the chloroplastic $^1O_2$-mediated cell death process, our current research aims to investigate the role of the photochemical reactions of photosynthesis and specifically the function of cyt f from the b6f complex, along with the subsequent events leading to cell death. The research reveals that a cellular state abundant in $^1O_2$ results in a reduction of photochemical processes in photosynthesis, leading to a decreased ATP pool due to the uncoupling of electron transport. Additionally, the process includes the release of cytochrome f at 30 minutes post light exposure to the plants, which occurs before a 12-hour dark treatment following rose Bengal application. It has been demonstrated that components of programmed cell death (PCD) are associated with proteasome proteins in one or several ways. One study indicated that E3 ubiquitin ligases are co-expressed with RPN9b (Wang et al., 2014); E3 ubiquitin ligases are essential for signaling substrates by marking them with an ubiquitin chain, a signal recognized by the 26S proteasome complex. In this research, computer-based network analysis indicates that components of the 26S proteasome, specifically RPN9b and RPN10, along with the E3 ubiquitin protein UPL5, are pivotal for the connection between cytochrome f and autophagy ATG8s (Chen & Hellmann, 2013; Furniss et al., 2018; Qiu et al., 2017; Rogov et al., 2023; Wang & Spoel, 2022). Analyzing network connections and protein-protein docking through focused sites reveals how Cyt f-proteasome proteins, E3 ubiquitin ligase, and components of programmed cell death (PCD) interact within the four crucial yet distinct systems of the cell, transmitting specific signals essential for maintaining cellular homeostasis.

3.Objectives

1. To identify Time & Strength of abiotic stress (Oxidative stress) required for induction of apoptosis like cell death.

2. To characterize linkage between Components of Photosynthetic machinery & oxidative stress induced signature of Programmed cell death.

3. To characterize the interacting partner of released cytochrome f (autophagy related) in cytosol to figure out sequence events leading to PCD.

4.Material and methods

Materials

Study was carried out on *Pisum sativum* plant due to ease in handling; better availability and shorter life cycle. For the capture of signature of PCD involved imaging tools, protoplasts system and whole plant system were used for the experiments.

Stress treatment

Oxidative stress were applied either to the whole plant (leaf)or to the isolated protoplasts. Rose Bengal (10µM) was sprayed onto 14 days young leaves and incubated in dark for 12hours and exposing into light for different time point (0,15,30,45,60,90) min.

Quantification of singlet oxygen:

Singlet oxygen quantified using fluorophore SOSG (Invitrogen) dye and observed using fluorescence microscopy. excitation/emission maxima at ~504/525 and fluorescence intensity of stain was analysed using Image J software.

Quantification of antioxidant molecule like carotenoids and tocopherol were quantified using (Kaisangsri et al., 2016; Thumthanaruk et al., 2021).

protoplasts were isolated from leaves of *Pisum Sativum* as described by Riazunnisa et al., 2007 with modification.

Quantification of cell death:

Cell death in whole leaf and isolated protoplasts was determined by dye exclusion method following (Ambastha et al 2017).

Signature of cell death:

Membrane blebbing:

Protoplasts isolated from pea plant leaves were subjected to oxidative stress (10µM Rose Bengal) under light (100 µ mol m^{-2} S^{-1}) and dark for 6 h. Samples were stained with Evans blue and observed under bright field of (Leica) at 100X magnification to identify blebs in the membrane.

DNA Ladder formation

DNA was extracted from whole plant system (leaf) at indicated time points after Rose Bengal treatment following CTAB method described by (Porebsk and Bailey et al. 1997) with modification and DNA-laddering was visualized on 1.8% Agarose gel.

Caspase 3 like activity assay:

Caspase 3 like activity was measured following method described by Bozhkov et al., (2004). leaf with control as well as treatment at different time points were ground in assay buffer supplied in kit and used according to manufacturer's instructions. DEVD-R110 (50mM), synthetic substrate for mammalian Caspase 3 was used as substrate.

Quantification of physiological parameter

Quantification of membrane leakage

Detached leaves from control as well as from Rose Bengal treated with different time interval of light illumination were incubated in deionized water for 15-20 min after incubation electrical conductivity measured by conductivity meter. this indicates membrane leakage in stress vs control condition. (Bajji, M. et al.2002)

Quantification of Total chlorophyll and chl a/b ratio:

Total chlorophyll content was quantified following Arnon (1949). One gram of finely cut fresh leaves were taken and ground with 20 – 40ml of 80% acetone.

Measurement of Photochemical reactions of photosynthesis:

Photochemical reactions of photosynthesis were measured in intact chloroplasts isolated from pea leaves untreated as well as treated with Rose Bengal, incubated in dark for 12h and exposed to light for different time points following method described by (Ambastha et al., 2020).

PSII Activity:

The PS2 activity of chloroplast was measured with a polarographic oxygen electrode (HANSATECH, UK), as described by Fufezan et al. (2002) with modification. The reaction mixture contained 50 mM Tricine-KOH (pH 7.0),5 mM $MgC1_2$, 1 mM $K_3Fe\ (CN)_6$, 10 mM 3-3,4-dichlorophenyl-1,1-dimethylurea (SIGMA-ALDRICH), and chloroplast suspension at a final concentration of 40µg Chl/ml.

PSI Activity:

The isolation of intact chloroplasts and measurement of PS1activity with an oxygen electrode were described by (Ou et al, 2013) with modification. The reaction mixture contained 50 mM Tris-HCl (pH 7.8),10 mM NaCl, 50 mM NH_4Cl, 50 mM MV,10 mM 3-3,4-dichlorophenyl-1,1-dimethylurea and chloroplast suspension equivalent to 40µg Chl/ml.

Whole chain electron transport reaction:

was measured by using50 mM Tris-HCl (pH 7.8),10 mM NaCl, 50 mM NH_4Cl,50 mM MV and chloroplast suspension equivalent to 40µg Chl/ml.

Characterization of cyt f:

Total chloroplast protein isolation:

Intact chloroplasts were isolated from control and untreated as well as RB treated leaves following method described by (Joly & Carpentier, 2011) Chlorophyll content of intact chloroplast was measured by recording the absorbance at 652 nm (Castle et al., 2011).

Fractionation of chloroplast protein:

Cytosolic and membrane protein extracts from pea leaves were prepared as described by (Debnam & Emes, 1999) with modification. Protein contents of the samples were determined by the Bradford assay using bovine serum albumin as a standard. The quality of the extracts was verified by immuno-blot using antibody.

SDS-PAGE and immunoblotting:

20 µg proteins prepared from membrane and matrix fraction of chloroplasts were subjected to 12% SDS-PAGE and transferred to PVDF membrane. Following transfer to PVDF, the proteins were visualized by Ponceau Stain (a.k.a Acid Red 112) (Sigma). Membrane blotting was performed with the Wet-system (Bio-Rad, USA) according to the manufacturer's instructions, The protein blots were blocked with 5% non-fat skimmed milk powder albumin in TBST buffer (20 mM Tris-HCl, pH 7.6, 0.8%NaCl, 0.1% Tween 20) and incubated with monoclonal anti-Cyt f antibody Anti-cyt f AS**20 4377** (Agrisera, Sweden) (Agrisera, dilution1:3000) for 2 h at room temperature. for housekeeping gene Anti-RbcS **AS07 259(dilution 1:5000) were used .** A peroxidase labelled anti-rabbit antibody (Agrisera, dilution1:25000) was used as a secondary antibody. The detection system is ECL Plus Western Blotting Reagents (Bio-Rad, USA)(Wang et al., 2014).

Protein interaction network analysis:

STRING database was used for interactome analysis. In the database multiple proteins option was selected and all the known key proteins were entered in the specific column. We have searched Cyt f, ATG8f, ATG8i, RPN9b pathways related search majorly. STRING gave us possible networking of proteins and based on our searched data we have found autophagy, Proteasome and ubiquitin linking pathways. *Arabidopsis thaliana* was used as reference for this study and based on *Arabidopsis thaliana* data we have generated and searched proteins for *Pisum sativum* (Szklarczyk et al., 2023).

Protein search and Homology Modelling :

The 3D structure of Cyt f was collected from Uniprot database; apart from that autophagy protein structures were not available in any database. Schrodinger maestro's homology modelling tool was used for generating autophagy protein' 3D structure. Templet (*Pisum sativum*) FASTA Sequence were collected and uploaded into homology modelling window and the preferred reference structure were selected from *Fabaceae* family. Computer generated 3D structures from the Alpha-fold database were downloaded (Geer et al., 2010; Johnson et al., 2008; Jumper et al., 2021; "UniProt: the universal protein knowledgebase in 2023," 2023). Both protein sequences were shown in multiple sequence viewer and aligned. Knowledge-based modelling was selected and the homology modelling program was run. The modelled structures was displayed, and for checking the structure quality, a protein reliability report and Ramachandran plot were generated (Zhu et al., 2014). In general, the three outputs (Steric Clashes', 'Missing Atoms' and 'Missing Loops') were looked at to determine the quality of a protein structure. If there was

any issue found; we have to run Loop refinement and performed protein preparation again. Again protein reliability report and Ramachandran plot were generated to check the prepared structure. Generated structure was used for further study (Sastry et al., 2013).

Sequence alignment and domain search:

The ATG sequences of plants are highly conserved, for this study first retrieved the ATGs of *Arabidopsis thaliana* and run the BLAST search against *Pisum sativum* ATGs using NCBI Protein BLAST (Ye et al., 2006). As well as after homology modelling again aligned the sequence using maestro Schrodinger's multiple sequence viewer and identified specific domains of proteins.

Protein-Protein Docking:

Protein network analysis results were used for selection of two protein partners. Cyt f and RPN9b structures were placed into protein-protein docking panel (Desta et al., 2020). In the receptor panel add A labelled Cyt f and B labelled RPN9b into the ligand panel. Attraction and repulsion data were added to the restrain column for both receptor and ligand and run the docking. These steps were performed for all our selected proteins.

Protein Interaction Analysis:

To identify the interaction between two proteins of interest, a protein interaction analysis method was employed. In addition to presenting counts of hydrogen bonds, salt bridges, disulphide bonds, and van der Waals clashes, the analysis finds residues in one protein that are within a specific distance of residues in another protein. Two proteins that desired to interface were chosen, labelled A and B, and their values were computed in the interaction panel.

5.Results

Singlet oxygen mediated cell death& PCD signature, physiological changes and Cytochrome f Status

To check the RB mediated singlet oxygen generation in pea leaves RB treated and dark incubated leaves were taken for measurement of singlet oxygen production after exposure to light for different time points indicated in materials and methods. Results suggest amplification in singlet oxygen species in a light exposure time dependent manner. To combat reactive oxygen

induced cellular damage, cells institute intrinsically regulated antioxidant system. Cell produces antioxidant enzymes against almost all reactive species of oxygen but cells do not have any antioxidant enzyme against singlet oxygen. However, some non-enzymatic molecules like carotenoids and tocopherol have ability to quench singlet oxygen species. To check antioxidant status in singlet oxygen rich environment, we checked the cellular carotenoid and tocopherol level in the leaves of pea seedlings under previously mentioned condition of treatment. Data shown suggest an increasing trend in the content of both the antioxidants up to light exposure of 30 min exposure beyond this time frame (45, 60 and 90 min) resulted in time dependent decrease in carotenoid and tocopherol content.

Singlet oxygen being very active reactive species has ability to damage subcellular components and macromolecules that eventually lead to cell death. The observation recorded advocate for an increase in cell death with increase in exposure time in both the systems. Further to classify singlet oxygen induced cell death, we looked for signature of PCD in the protoplasts treated with RB and exposed to light for different time points as indicated in materials and methods. Results shows appearance of membrane blebs in the RB treated protoplasts exposed to light for 30 min. further light exposure to the protoplasts leads to increase in the number of blebs. Earlier studies have shown that during execution process of PCD some endonucleases get activated and they cleave DNA at the point of H1 to give rise of DNA fragment of 200bp that eventually forms a ladder on gel. To visualize DNA ladder during singlet oxygen induced PCD in our system, we prepared DNA from the leaf samples harvested after each time point of light exposure and separated on 1.8% agarose gel. Results indicate appearance of DNA ladder in the leaf samples harvested at 30 min of light exposure that became prominent in the samples harvested at 45 min. Further photo exposure led to disappearance of DNA ladder. Caspase activation has been reported as one of the key steps in PCD activation in animal cells. Although caspases are absent in the plants but caspase 3 like activity has been reported in plant cells undergoing PCD process. When we tested our system for caspase 3 like activity, we found caspase 3 like activity in all the samples collected at each and every time point of light exposure and increased in time dependent manner .

After having confirmed the cell death induced by singlet oxygen is PCD like cell death, we analysed status of some physiological events mainly related to chloroplasts during singlet oxygen rich condition. We measured membrane leakage, pigment composition of chloroplasts and photochemical reactions of photosynthesis in the pea seedlings treated with RB incubated in dark and exposed to light for 15, 30, 45, 60 and 90 min. Results shows the pattern of membrane

leakage as recorded for change in electrical conductivity, quantity of chlorophyll a and chlorophyll b and ratio of Chl a/b. Membrane leakage a measure of electrical conductivity showed significant increase in the leaf discs collected within 15 min of light exposure, further increase in light exposure resulted in higher electrical conductivity. At 90 min of light exposure membrane became very leaky as evident from very high (17000µs) conductivity. Observations made on pigment composition with chloroplasts during high singlet oxygen environment showed decrease in the both chlorophylls *a* and *b* in accordance with duration of photo-exposure. While a less decrease in chlorophyll a content has been recorded in the leaf samples treated with RB incubated in dark and exposed to light, rate of decrease in chlorophyll *b* content was considerably high. For example at 45 min of light exposure a time point where hallmarks of PCD became detectable Chlorophyll a content was reduced by 27% and at the same time point of light exposure chlorophyll b content was dropped by 45%.

After characterizing macromolecular composition of chloroplast and membrane, photochemical reactions of photosynthesis was characterized polarographically using oxygen electrode (Hansatek UK). Results suggest a significant decline in electron transport across PSII, PSI and whole chain electron transport system in a time dependent manner of light exposure to the broken chloroplasts prepared from the leaves of pea seedlings sprayed with RB and incubated in dark for 12h. While there was a sharp and significant decrease in electron transport through PSII, PS I and whole chain electron transport showed a significant but lesser decrease in electron transport activity compared to decrease recorded in PSII. For example PS II activity in the chloroplasts isolated from RB treated plants and photo-exposed for 30 and 45 min had shown a decrease in activity by 25% and 32% respectively, whereas at the same treatment decrease in PSI activity was recorded to be 15 and 18% and decrease in electron transport through whole chain was 18 and 23%.

Computer Based Analysis

Protein network analysis showed some interesting results about interaction between autophagy protein ATG8 isoforms, 26S proteasome subunits RPN9b and RPN10, and E3 ubiquitin ligase UPL5 protein. Analysis indicates that ATG8i, ATG8f, ATG8a, and ATG8b participate via RPN10 in Arabidopsis.Since *Pisum sativum* lacks homologues for ATG8a and ATG8b (Johnson et al., 2008), we have performed interaction analysis with only ATG8f and ATG8i Data shown, only UPL5 is found to be necessary and actively connected to the RPN10 and/or ATG8f; other than that none of the family members of E3 ubiquitin ligases were participated directly with

proteasomal member proteins and autophagic member proteins (Szklarczyk et al., 2023; Wang et al., 2014).

Homology modelling was carried out for five important *Pisum sativum* proteins, namely RPN9b, RPN10, UPL5, ATG8i, and ATG8f. There are some unfavourable bond angle deviations specifically found in RPN10 and UPL5, so we had done loop refinement and improved the structure quality; confirmed the structure quality of protein models by the Ramachandran plot, and protein reliability reports showed us favourable models (Zhu et al., 2014).

Arabidopsis thaliana and *Pisum sativum*'s sequence alignment provided an insight into the specific domain site identification in *Pisum sativum*.

Protein-protein interaction data gives an insight into specified domain site binding potential of the two linking proteins.

6.Discussion

This study provides a detailed investigation into the amplification of reactive oxygen species (ROS), particularly singlet oxygen ($^1O_2$), in pea seedlings treated with Rose Bengal (RB). Your results highlight the photodynamic properties of RB and its ability to generate singlet oxygen upon light exposure, leading to oxidative stress and cell death through a mechanism resembling programmed cell death (PCD).

Here's a breakdown of the key findings and their implications:

1. Singlet Oxygen Generation and its Effect on Pea Seedlings

- **Rose Bengal Treatment**: Pea seedlings were treated with an aqueous solution of Rose Bengal (10 μM) and incubated in the dark to accumulate protochlorophyllide. This intermediate in the chlorophyll biosynthetic pathway generates singlet oxygen upon light exposure.
- **Light Exposure Effects**: Upon light exposure, the seedlings showed visible changes after 45 minutes, including yellowing and curling of the leaves, ultimately leading to cell death (though the specific timeline is not provided). These findings confirm that Rose Bengal, as a photodynamic dye, effectively generates singlet oxygen under light exposure.
-

2. Singlet Oxygen-Induced Cellular Effects

- **Antioxidant Response**: As singlet oxygen accumulates, pea seedlings do not have specific enzymatic systems to neutralize it. Instead, non-enzymatic antioxidants like tocopherol and carotenoids play a role in quenching singlet oxygen. Initially, these antioxidants increased in response to singlet oxygen, but beyond 30 minutes of light exposure, their levels declined, suggesting a limited capacity of the antioxidant machinery to cope with sustained oxidative stress.
- **Physiological Changes**:
    - **Membrane Conductivity**: Membrane integrity was compromised, as indicated by increasing electrical conductivity, a sign of membrane leakage.
    - **Chlorophyll Degradation**: Chlorophyll content, particularly chlorophyll b, was significantly reduced, which is typical of oxidative damage in plants.
    - **Photosynthetic Activity**: Electron transport across PSII was particularly sensitive to singlet oxygen-induced oxidative stress, with a significant reduction in ATP production and other key photosynthetic activities.

3. PCD-Like Characteristics Induced by Singlet Oxygen

- **PCD Indicators**: The study observed key features of PCD, including:
    - **Membrane Blebbing**: A hallmark of cellular stress and PCD.
    - **DNA Ladder Formation**: The appearance of DNA fragmentation indicative of PCD.
    - **Caspase-3-Like Activity**: Although plants lack true caspases, caspase-3-like activity was detected, which is consistent with plant PCD processes.

These findings suggest that singlet oxygen accumulation induced PCD-like responses, with a moderate level of ROS driving cells toward PCD rather than necrosis.

4. Mitochondrial and Chloroplast Involvement in PCD

- **Mitochondria in PCD**: Previous research has documented the role of mitochondria in executing PCD in plants, particularly through the release of cytochrome c (cyt c). The

study highlights the role of ROS in triggering the opening of the mitochondrial permeability transition pore (PTP), which contributes to cell death.

- **Chloroplasts as ROS Sources**: Chloroplasts, in addition to mitochondria, were also found to release cytochrome f (cyt f) during oxidative stress. This suggests that chloroplasts may contribute to PCD in response to singlet oxygen accumulation, which has been less explored in the context of ROS-mediated plant death.

5. Protein-Protein Interactions and Proteasome/Autophagy Involvement

- **Cytochrome f Release and Downstream Events**: The study examined the release of cytochrome f during singlet oxygen-induced oxidative stress and tested the downstream signaling pathways. Computational and protein network analyses revealed that cyt f interacts with components of the proteasome (RPN9b, RPN10) and autophagy (ATG8f). Specifically, the E3 ubiquitin ligase UPL5 was found to play a critical role in connecting these pathways.
- **Proteasomal and Autophagic Pathways in PCD**: This suggests that the proteasome and autophagy systems are likely involved in executing the PCD triggered by singlet oxygen, following cytochrome f release. These findings may provide insights into how plants manage cellular damage and stress under oxidative conditions.

7.Conclusion and summary

Conclusion

In this study, we investigated the role of **cytochrome f** during singlet oxygen-induced programmed cell death (PCD) in pea seedlings. Singlet oxygen, generated by **Rose Bengal (RB)** treatment and light exposure, led to oxidative stress, triggering PCD signatures such as membrane blebbing, DNA ladder formation, and caspase-3-like activity. Antioxidant defense molecules like **tocopherol** and **carotenoids** were overwhelmed, indicating the inability of the cells to mitigate the oxidative damage. Additionally, chloroplast functions, especially **photosystem II (PSII)**, were significantly impaired. Immunoblotting revealed the translocation of **cytochrome f** from chloroplast membranes to the cytosol (soluble fraction), supporting its role in PCD. Computational studies further showed that **cytochrome f** interacts with the **proteasome**

and **autophagy** systems, which are involved in the downstream execution of PCD. This study highlights the involvement of **cytochrome f**, the proteasome, and autophagy in the execution of PCD under singlet oxygen stress in plants.

Summary

In this study, singlet oxygen was generated in 14-day-old pea seedlings by treating leaves with 10 μM Rose Bengal, incubating them in dark for 12 hours, and exposing them to light for varying durations (15, 30, 45, 60, 90, and 120 minutes).

1. Singlet oxygen production, detected by SOSG fluorescence, increased significantly compared to control and unchallenged leaves.
2. Maximum singlet oxygen levels were observed after 45 minutes of light exposure, at which point antioxidants like carotenoids and tocopherols showed a remarkable decrease.
3. Programmed cell death (PCD) hallmarks, including membrane blebbing, DNA laddering, and caspase-3-like activity, were observed at 45 minutes of light exposure in both whole leaves and protoplasts.
4. Cellular ROS burden at this time point significantly impacted physiological functions, such as membrane leakage, chlorophyll content, pigment ratio, and photosynthetic reactions.
5. Cytochrome f (Cyt f) translocated from the membrane to the cytosol after 45 minutes of light exposure.
6. Cyt f released during PCD interacted with components of the ubiquitin pathway and/or autophagy system, suggesting its role in the downstream execution of PCD.
7. Overall, our findings highlight the significant role of **cytochrome f** in singlet oxygen-mediated cell death in pea seedlings.

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9.Paper Published/communicated

- Bhatt, M., Pandey, S. S., Tiwari, A. K., & Tiwari, B. S. (2021). Plastid-mediated singlet oxygen in regulated cell death. *Plant biology (Stuttgart, Germany)*, *23*(5), 686–694. https://doi.org/10.1111/plb.13260
- Ombale, S., Bhatt, M., Tiwari, A.K., Sharma, A., &Tiwari , B.S.(2024).Nitro-oxidative burden in the plants& their survival through regulated cell death.Protoplasma (under revision).
- Bhatt, M., Bhatt, R., Tiwari, B.S. Role of cytochrome f during singlet oxygen induced programmed cell death in Pisum sativum. Physiologia Plantarum (under review).

10. Conference /symposia attended:

- Five Days hands on workshop of microscopy at INSTITUTE OF ADVANCED RESEARCH GANDHINAGAR
- National symposium on Basic And translational clinical research in cancer biology 2019 at INSTITUTE OF ADVANCED RESEARCH GANDHINAGAR
- Poster presentation at Research conclave 2021 at INSTITUTE OF ADVANCERESEARCH,GANDHINAGAR
- Oral presetation at Research conclave 2023 at INSTITUTE OF ADVANCED RESEARCH,GANDHINAGAR.
- National webinar on “Plant Biotechnology for sustainable Agriculture "Organized by SOCIETY FOR PLANT RESEARCH.
- International e-conference on “Multifaceted Application in Biotechnology &Applied science” (e-COMBAS 1.0) 2024.